CLONING AND HETEROLOGOUS EXPRESSION OF *CRYPTOCOCCUS NEOFORMANS CnSRB1* cDNA IN *SACCHAROMYCES CEREVISIAE*

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Abstract. In this study, we report the results of cloning, sequencing and functional analysis by complementation test of the putative *Cryptococcus neoformans* homolog *CnSRB1*. The nucleotide sequence revealed 63% identity, and the deduced amino acid sequence showed 66 and 64% identity to its respective homolog of *Saccharomyces cerevisiae* and *Candida albicans*, respectively. Functional complementation test indicated that the putative *CnSRB1* gene could compensate the defect caused by a mutation in *ScSRB1* in the *S. cerevisiae srb1* mutant. Taken together, these results suggest that the putative CnSrb1p is a functional homolog of ScSrb1p.

INTRODUCTION

Cryptococcus neoformans (*C. neoformans*), a basidiomycetous encapsulated yeast, has become a wide-spread opportunistic human pathogen. It causes life-threatening meningitis, which is fatal if not treated effectively especially in immunocompromised patients due to HIV infection, cancer chemotherapy or transplantation surgery (Mitchell and Perfect, 1995; Ampel, 1996; Baron, 1996; Casadevall and Perfect, 1998; Saubolle, 1998; Sukroongreung *et al*, 1999). Three major varieties of the yeast pathogen have been identified. *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* are distributed worldwide and are the major causes of human disease. The less common variety, *C. gattii*, has been isolated from eucalyptus trees in tropical and sub-tropical regions (Casadevall and Perfect, 1998).

The polysaccharide capsule surrounding yeast cell wall is a major virulence factor. Its main constituents are a high molecular weight polysaccharide, glucuronoxylomannan (GXM), representing 88% of the total capsular weight, and galactoxylomannan (GalXM) and some mannoproteins representing the remaining 12% (Baron, 1996; Bose *et al*, 2003, Janbon, 2004). Mutants deficient in GXM are avirulent in mice (Chang and Kwon-Chung, 1994). GXM has a branched structure with monosaccharide branches of xylose and glucuronic acid linked to a mannose backbone (Cherniak *et al*, 1998). Synthesis of GXM requires the nucleotide-activated sugars GDP-mannose and UDP-xylose, and UDP-glucuronic acid is also required. GDP-mannose biosynthesis is a critical early step, requiring sequential actions of phosphomannose isomerase (PMI), phosphomannomutase (PMM) and GDP-mannose pyrophosphorylase (GMP or GDP-mannose synthase or mannose-1-phosphate guanyl transferase). These enzymes are highly

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conserved in several fungi. So far, only the gene encoding PMI (*MAN1*) has been identified and cloned from *C. neoformans* (Will *et al*, 2001).

The gene encoding GMP has been cloned and characterized in several yeast species, including *Saccharomyces cerevisiae* (*ScSRB1/ScPSA1/ScMPG1/ScVIG9*) (Stateva *et al*, 1991; Benton *et al*, 1996; Hashimoto *et al*, 1997) and the human fungal pathogens *Candida albicans* (*CaSRB1/CaVIG9*) and *Candida glabrata* (*CgVIG9*) (Warit *et al*, 1998,2000; Warit, 1999; Ohta *et al*, 2000). The genes are essential for viability and mutations of the gene or conditional down-regulation of its transcription cause severe morphological abnormalities and ultimately death as a result of loss of cell wall integrity in both *S. cerevisiae* and *C. albicans* (Warit, 1999; Zhang *et al*, 1999a, b; Tomlin *et al*, 2000; Warit *et al*, 2000, Yoda *et al*, 2000). Recently, homologs have been cloned and characterized from the filamentous fungus *Trichoderma reesei* (Zakrewska *et al*, 2003) and the parasitic protozoa *Leishmania mexicana* (Garami and Ilg, 2001; Garami *et al*, 2001). In the latter, GDP-mannose biosynthesis is not essential for viability in culture, but constitutes a major virulence factor.

In this paper, we report the cloning, sequencing and analysis of the putative *C. neoformans SRB1* (*CnSRB1*) and indicate that it is a functional homolog of *ScSRB1* because of its ability to partially complement the defect of *S. cerevisiae srb1* mutant.

MATERIALS AND METHODS

Organisms, plasmids and media

The organisms and plasmids used in this study are shown in Table 1. Standard yeast rich (YEPD) and minimal media (SD: 2% glucose, 0.67% YNB without amino acids) with appropriate supplements were used as reported by Burke *et al* (2000). Bacterial strains were grown in LB with and without 100 μg/ml ampicillin (Sambrook and Russell, 2001).

Yeast genomic DNA extraction and Southern blot analysis

Genomic DNA of *C. neoformans* grown in YEPD at 30ºC for 48 hours was isolated by glass bead preparation method (Burke *et al*, 2000). Approximately 10 μg of genomic DNA was completely digested with appropriate restriction enzymes (Biolabs) and analyzed by Southern hybridization using the non-radioactive DIG-system (Boehringer Mannheim, Germany).

Table 1
Organisms and plasmids used in the study.

Name	Characteristics	Sources
Organism		
E. coli DH5-alpha	*supE*44 ΔlacU169 (Φ80 lacZ ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Hanahan (1983)
C. neoformans B-4500	MATα serotype D	Clinical isolate
S. cerevisiae 7SLU	*MATα srb1-1 leu2 ura3*	Stateva *et al* (1991)
Plasmid		
pUC-CaSRB1	Derived from pUC19 containing *CaSRB1* clone A DNA fragment.	Warit (1999)
pGEMT-CnSRB1	Derived from pGEM-T easy vector containing the putative full-length of *CnSRB1* cDNA fragment.	This study
p*PGK*-CnSRB1	Derived from p*PGK*-YES2 containing the putative full-length of *CnSRB1* cDNA fragment.	This study

RNA extraction and cDNA synthesis

RNA was extracted from yeast grown in 50 ml of YEPD media at 30ºC for 24 hours by Brown's method (1994). In brief, after washing in 5 ml of cold RNA extraction buffer (0.1M LiCl, 0.1M Tris-HCl, pH 7.5, 0.01 M DL-dithiothreitol), *C. neoformans* cells were immediately vortexed with 14 g of glass beads (0.4 mm diameter), 5 ml of cold RNA extraction buffer, 10 ml of phenol/chloroform solution (1:1 v/v) and 1 ml of 10% SDS for 5 minutes. The cell mixture was centrifuged at 10,000 rpm for 5 minutes at 4ºC. The upper aqueous phase was extracted with 5 ml of phenol/chloroform solution once and with chloroform twice. After that, RNA was precipitated by adding 0.1 volume of 3M Sodium acetate, pH 5.2 and 2 volumes of absolute ethanol and incubated at -20ºC overnight. Next day, RNA was re-precipitated again by using 2 ml DEPC-treated water, 700 μl of 3 M Sodium acetate solution pH 5.2 and 4.2 ml of absolute ethanol and stored at -20ºC overnight. Finally, the RNA was collected, treated with DNase I (Biolabs), dissolved in 100 μl of sterilized RNase-free water and kept at -70ºC until further use. Synthesis of cDNA was performed at 42ºC for 2 hours in a total volume of 40 μl containing 2.5 μg of total RNA, 1 μl of fresh 0.1M DL-dithiothreitol, 2 μl of 10 mM dNTPs, 1 μl of 500 μg/ml oligo $(dT)_{15}$, 1 μl of 500 μg/ml random hexamer, 0.2 μl of Rnasin Rnase inhibitor, 1x reverse transcription buffer and 8 units of AMV-RT enzyme (Promega).

Northern blot analysis

RNA was electrophoresed under denaturing conditions, transferred to charged modified Nylon membrane (Sigma) according to the method of Sambrook and Russell (2001). Northern blotting and hybridization protocols of the non-radioactive DIG-system were followed as recommended by the manufacturer (Boehringer Mannheim, Germany).

PCR amplification of the putative *CnSRB1* gene

In a volume of 50 μl, five-fold dilution of cDNA was amplified with a standard PCR mixture for Vent DNA polymerase (BioLabs) and primers CnSRB7 (5′-CGGGAGCCATGAA GGCTCTGATCC-3′) and CnSRB8 (5′-GCTC TAGATTACATAACAATACGGGGC-3′) specific for the conserved regions at the 5′- and 3′-ends of *ScSRB1*, *CaSRB1* and putative *CnSRB1* identified from *Cryptococcus* genome database. PCR amplification consisted of an initial denaturation at 95ºC for 5 minutes, followed by 30 cycles of denaturation at 95ºC for 1 minute and annealing/elongation at 72ºC for 2 minutes.

Plasmid constructions for DNA sequencing and functional complementation

For sequencing, PCR product was cloned into pGEM-T Easy plasmid (Promega) using the conditions recommended by the manufacturer. Positive clones containing pGEM-CnSRB1 were selected and the extracted plasmid was sequenced using primers CnSRB9 (5′-ACCGAACCTCTCGGCACTGCG-3′), CnSRB10 (5′-TGATGGTTGACCCTTCTG CGGAA-3′), CnSRB11 (5′-CTGGAGACGG ACACCAGGCC-3′) and T7 promoter primers.

For the functional complementation test, the putative *CnSRB1* cDNA gene from plasmid pGEM-CnSRB1 was cloned under the control of phosphoglycerate kinase promoter by ligation into p*PGK*-YES2 at *Not*I and *Xba*I cloning sites, resulting in a recombinant plasmid, p*PGK*-CnSRB1. The p*PGK1*-YES2 plasmid, kindly given by Walid Omara (University of Manchester, UK), was constructed as follows. A fragment of the vector pYES2 (*In*Vitrogen) containing p*GAL1* (from nucleotide position 1 to 450) was excised using *SpeI* and the remaining large *SpeI* fragment, after dephosphorylation and purification, was ligated to a 465 bp p*PGK1* containing fragment, amplified from *S. cerevisiae* genomic DNA. The primers used for the p*PGK1* amplification were 5′-CGGACTA GTCTGAAAAAACCCAGACACGC-3′ and 5′-CGGACTAGTTGTAAAAAGTAGATAATTAC-3′.

Functional complementation test in *S. cerevisiae srb1* mutant

S. cerevisiae 7SLU strain (Table 1), containing mutated *ScSRB1* gene, was transformed with either p*PGK1*-YES2 or p*PGK1*-CnSRB1 plasmid. Transformants were grown on appropriately supplemented minimal media with and without 10% sorbitol.

Bacterial and yeast transformation

E. coli DH5-alpha was transformed by heat shock method (Sambrook and Russell, 2001) and *S. cerevisiae* 7SLU was transformed by the method recommended by Stateva *et al* (1991). In short, *S. cerevisiae* 7SLU cells were collected from 4 ml of culture grown in rich media plus 10% sorbitol (w/v) at 30ºC until the OD_{600} reached 0.5. Then yeast cells were washed in TES buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 % sorbitol) once and re-suspended in 200 μl of TES buffer. Approximately 10 μg of plasmid DNA were added, gently mixed and incubated at 30ºC for 30 minutes. After that, an equal volume of freshly prepared 70% polyethylene glycol-400 (PEG-400) solution was mixed and incubated further at 30ºC for 60 minutes. PEG was removed by washing with 5 ml of 10% sorbitol and the cells were suspended in 500 μl of 10% sorbitol. Finally, 50-100 μl of the cell suspension were spread on selective media with appropriate supplements.

Sequence analysis

All nucleotide sequencing and primer synthesis were conducted at BioService Unit, NSTDA, Bangkok, Thailand. Sequence homology analysis was performed using standard web base tools (http://www.yk.rim.or.jp/~aisoai/tool.html). DNA sequence was converted into amino acid sequence using standard codon usage (http://arbl.cvmbs.colostate.edu/molkit/translate/index.html) and aligned with CLUSTALW program (http://www.ebi.ac.uk/clustalw/) using TIGR's *Cryptococcus neoformans* genome database (http://www.tigr.org/tdb/e2k1/cna1/). Phylogenetic tree was constructed using un-weighted pair group method with arithmetic averages (UPGMA) and maximum parsimony (MP) methods, implemented in the PAUP version 4.0 b1 software, on the basis of the homologous protein sequences obtained from the CLUSTALW alignment.

RESULTS

Analysis of putative *SRB1* homolog

The *CnSRB1* cDNA sequence, 1,095 nucleotides in length, was identified by BLAST search of the TIGR *C. neoformans* genome database using the nucleotide and protein sequences of *SRB1* of *S. cerevisiae* (*ScSRB1*) and *C. albicans* (*CaSRB1*) as queries. Primers CnSRB7 and CnSRB8 were then used to amplify the putative *CnSRB1* cDNA from total RNA of *C. neoformans* strain B-4500 by RT-PCR. Only a single 1-kb-long product was obtained (Fig 1a) and cloned. Southern hybridization of genomic DNA digested with either *Pst*I, *Eco*RI or *Bgl*II (Fig 1b) and Northern hybridization of total RNA of *C. neoformans* (Fig 1c) using *CnSRB1* as probe resulted in a single hybridized band. This suggests that *CnSRB1* has no paralog in the genome of *C. neoformans*. The DNA sequence of the cloned *CnSRB1* (Genbank accession DQ116945) is identical to that of the previously predicted CDS in *C.neoformans* var. *neoformans* JEC21 (Genbank accession XM_569600).

Comparative analysis of *SRB1* sequences

The nucleotide sequence of the putative *CnSRB1* CDS was aligned to its respective *S. cerevisiae* and *C. albicans* homologs using CLUSTALW revealing 63% nucleotide sequence identity between them. Similar levels of identity were revealed for the respective protein sequences, with CnSrb1p being 64% and 66% identical to its homologous protein in *S. cerevisiae* and in *C. albicans*, respectively. Four amino acids occupying positions

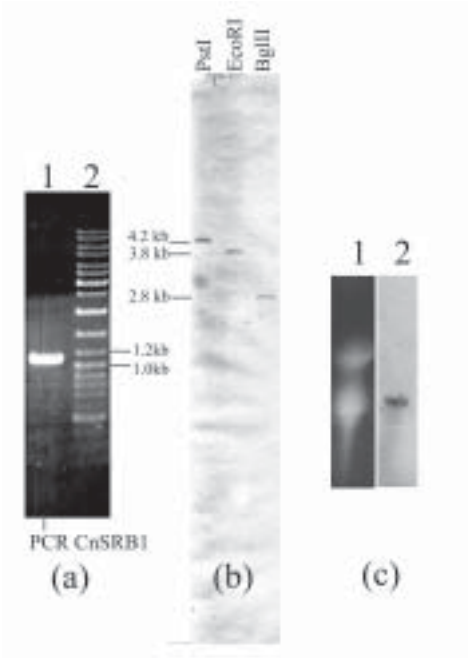

Fig 1–(a) The putative *CnSRB1* cDNA product after RT-PCR amplification using primers CnSRB7 and CnSRB8 of total RNA of *C. neoformans* strain B-4500. Lane1, RT-PCR product with a size of about 1 kb; lane 2, DNA size markers. (b) Southern hybridization of *C.neoformans* genomic DNA digested with restriction enzyme as indicated and probed with *CnSRB1* cDNA. (c) Northern hybridization of total RNA of *C. neoformans* B-4500 with *CnSRB1* cDNA probe. Lane1, image of UV-exposed total RNA on the membrane; lane 2, hybridization signal obtained.

154 and 251-253 in the putative CnSrb1p are not present in ScSrb1p and CaSrb1p, unlike that at position 257 which is present in both of CnSrb1p and CaSrb1p, but not in ScSrb1p. Moreover, codon usage of CnSrb1p was different from those of *ScSRB1* and *CaSRB1*. For example, in *CnSRB1* the preferred codon for leucine, proline and glutamine is CUC, CCC and GAG respectively, while in *ScSRB1* and *CaSRB1* these codons are used 10 times less. Arginine is encoded in *C. neoformans* preferentially by CGU, CGC, CGA and CGG and only infrequently by AGA, the preferred codon in both *S. cerevisiae* and *C. albicans*.

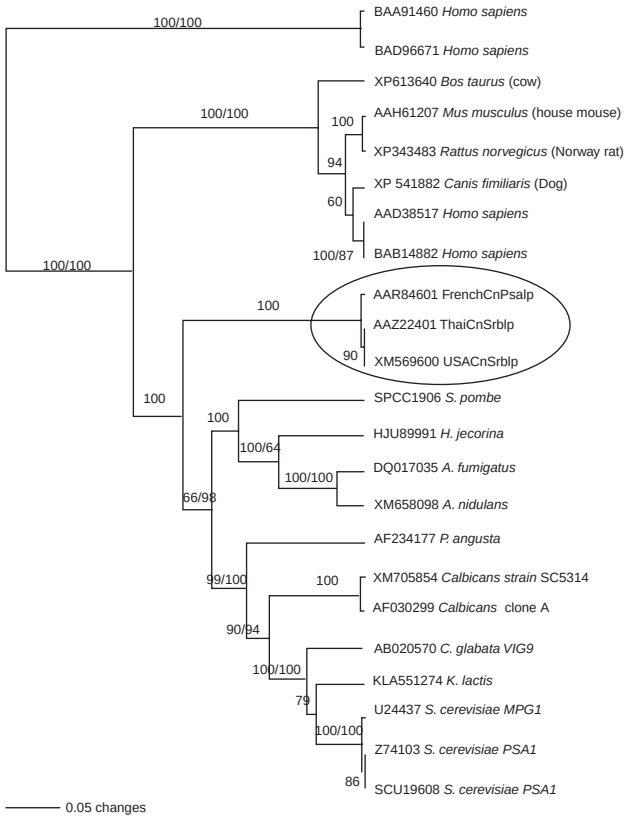

Fig 2–Phylogenetic tree showing the relationship of Srb1p of basidiomycetous, ascomycetous fungal and mammal species. A circle indicates the basidiomycetous fungal group. The tree was generated by PAUP version 4.0 b1 software using the results of homologous protein sequences aligned with the CLUSTALW program. The numbers on the branches represent bootstrapping values of unweighted pair group method with arithmetic averages (UPGMA) and maximum parsimony (MP) method respectively. Organism source and NCBI database accession number of each sequence are as indicated. Srb1p sequence of the three *C. neoformans* isolates from France, Thailand and United State is from NCBI accession AAR84601, AAZ22401 and XP_569600, respectively.

A phylogenetic tree of proteins homologous to CnSrb1p was constructed (Fig 2), using the sequences obtained from NCBI (http://www.ncbi.nlm.nih.gov). CnSrb1p is very

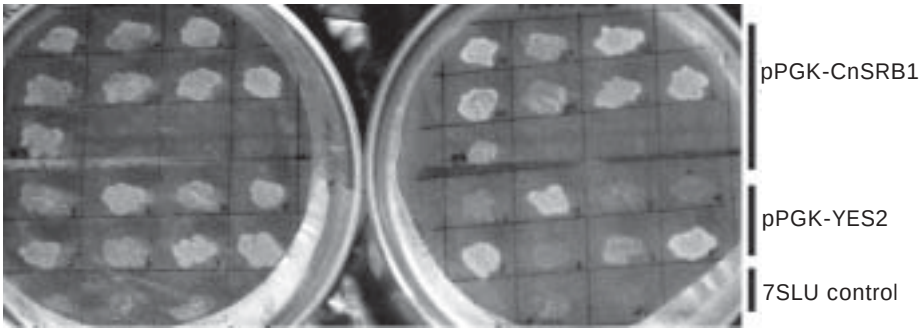

Fig 3–Growth of *S. cerevisiae srb1* 7SLU strain (*srb1*^{D276} strain) containing either p*PGK*-YES2, p*PGK*-CnSRB1 or none. Yeasts were grown on solid selective media (SD) containing leucine (Leu) with and without 10% sorbitol (Srb) as indicated.

similar (63-72%) to homologs of other ascomycetes including *S. cerevisiae*, *C. albicans*, *Hypocrea jecorina*, *Aspergillus fumigatus*, *Aspergillus nidulans*, *Schizosaccharomyces pombe*, *Candida glabrata*, *Kluyveromyces lactis* and *Pichia angusta*. The major differences in predicted amino acid sequences of CnSrb1p in comparison to those from other yeast species can be found at positions 240 to 260. Similarities to mammalian homologs such as those of mouse, cow, dog, rat and human were lower (20-58%). The phylogenetic tree demonstrates that the GMP homologs are clearly classified into 3 groups. The putative CnSrb1p is similar to basidiomycetes and different from ascomycetes and mammals.

Function of the putative *CnSRB1* cDNA in *S. cerevisiae* mutant

S. cerevisiae 7SLU strain contains a mutated *ScSRB1* gene leading to dependence on an osmotic stabilizer (10% sorbitol) for growth and protection against lysis by osmotic shock. After transformation with either p*PGK*-CnSRB1 or p*PGK*-YES2, 100 colonies of each, as well as the wild type 7SLU strain, were grown on minimal agar with or without 10% sorbitol. All *S. cerevisiae* 7SLU mutants harboring p*PGK*-CnSRB1 grew normally on minimal media either with or without 10% sorbitol. In contrast, only about 22% of *S. cerevisiae* 7SLU and 7SLU transformants containing p*PGK*-YES2 were able to grow on the agar without 10% sorbitol, as shown in Fig 3. This indicates that *CnSRB1* cDNA present in p*PGK*-CnSRB1 was able to functionally compensate the mutated *ScSRB1* in the 7SLU strain. That some of the untransformed 7SLU cells as well as the transformants containing the control vector p*PGK*-YES2 could survive on the media without sorbitol was the result of the high level of reversion of the sorbitol-dependent phenotype in 7SLU.

DISCUSSION

We have cloned and sequenced the putative *SRB1/PSA1/MPG1/VIG9* homolog of *C. neoformans* strain B-4500 (*CnSRB1*). The function of the gene was studied by complementing *S. cerevisiae srb1* mutant 7SLU strain.

The *srb1* 7SLU strain is *srb1*^{D276} strain expressing ScSrb1p with a single mutation Gly276Asp and showing a sorbitol-dependence phenotype (Tomlin *et al*, 2000). Our finding shows that *CnSRB1* can compensate the defect in 7SLU strain, suggesting that the cloned gene is a functioning *SRB1*. Moreover, *in silico* search of the *C. neoformans* genome revealed no other paralog to *CnSRB1*.There

is only a single *CnSRB1* gene predicted in the genome of *C. neoformans* var. *neoformans* strain JEC21 (Loftus *et al*, 2005), located on chromosome 3, containing 12 exons and 11 introns. Only one of the introns contains a terminal sequence of 5′AT...AC3′ with the length of 53 nucleotides, whilst the remaining ten have the terminal sequences of 5′GT...AG3′ with lengths of 46-106 nucleotides. Both of the Southern and Northern hybridization using the *CnSRB1* gene as a probe, as well as RT-PCR with *CnSRB1* specific primers confirmed that there is only a single gene in the *C. neoformans* genome. The sequence of CnSrb1p also contains a glycine at amino acid residue 276. There are similar reports of successful complementation of single amino acid-substituted mutants *vig9-1* and *vig9-2*, which are also osmotically fragile, by wild type *ScSRB1* gene (Yoda *et al*, 2000).

In summary, *CnSRB1* cDNA has been cloned and shown to have the ability to complement the function of *S. cerevisiae srb1* mutant strain. Due to the importance of GMP, it is likely that inhibition of GMP would lead to fungal growth arrest or cell death. The cloned CnSRB1 will therefore be useful for production of GMP, which can be used to formulate target-based screening of new anti-cryptococcal compounds.

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REFERENCES

Ampel NM. Emerging disease issues and fungal pathogens associated with HIV infection. *Emerg Infect Dis* (Synopses) 1996; 2: 109-16.

Baron S. Mycology. In: Medical microbiology. 4th ed. Texas: The University of Texas Medical Branch at Galveston, 1996.

Benton BK, Driscoll-Plump S, Roos J, Lennarz W, Cross F. Overexpression of *Saccharomyces cerevisiae* G1 cyclins restores the viability of *alg1 N*-glycosylation mutants. *Curr Genet* 1996; 29: 106-13.

Bose I, Reese AJ, Ory JJ, *et al*. A yeast under cover: the capsule of *Cryptococcus neoformans*. *Eukaryot Cell* 2003; 2: 655-63.

Brown AJP. The method of RNA extraction and mRNA quatitation in *Candida albicans*. In: Maresca B, Kobayashi GS, eds. Molecular biology of pathogenic fungi: A laboratory manual. New York: Telos Press, 1994: 127-34.

Burke D, Dawson D, Stearns T. Methods in yeast genetics (A Cold Spring Harbor Laboratory Course Manual). New York: Cold Spring Harbor Laboratory Press, 2000.

Casadevall A, Perfect JR. *Cryptococcus neoformans*. Washington DC: American Society for Microbiology Press, 1998.

Chang YC, Kwon-Chung KJ. Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. *Mol Cell Biol* 1994; 14: 4912-19.

Cherniak R, Valafar H, Morris LC, Valafar F. *Cryptococcus neoformans* chemotyping by quantitative analysis of 1H nuclear magnetic resonance spectra of glucuronoxylomannans with a computer-stimulated artificial neural network. *Clin Diagn Lab Immunol* 1998; 5: 146-59.

Garami A, Ilg T. Disruption of mannose activation in *Leishmania mexicana*: GDP-mannose pyrophosphorylase is required for virulence, but not for viability. *EMBO J* 2001; 20: 3657-66.

Garami A, Mehlert A, Ilg T. Glycosylation defects and virulence phenotypes of *Leishmania mexicana* phosphomannomutase and dolicholphosphate mannose synthase gene deletion mutants. *Mol Cell Biol* 2001; 21: 8168-83.

Hanahan D. Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* 1983; 136: 557-80.

Hashimoto H, Sakakabara A, Yamasaki M, Yoda K. *Saccharomyces cerevisiae VIG9* encodes

GDP-mannose pyrophosphorylase, which is essential for protein glycosylation. *J Biol Chem* 1997; 272: 16308-14.

Hull CM, Heitman J. Genetics of *Cryptococcus neoformans*. *Annu Rev Genet* 2002; 36: 557-615.

Janbon G. *Cryptococcus neoformans* capsule biosynthesis and regulation. *FEMS Yeast Res* 2004; 4: 765-71.

Loftus BJ, Fung E, Roncaglia P, *et al*. The genomes of basiomycetous yeast and human pathogen *Cryptococcus neoformans*. *Science* 2005; 307: 1321-4.

Mitchell TG, Perfect JR. Cryptococcosis in the era of AIDS-100 years after the discovery of *Cryptococcus neoformans*. *Clin Microbiol Rev* 1995; 8: 515-48.

Ohta A, Chibana H, Arisawa M, Sudoh M. The *VIG9* gene products from the human pathogenic fungi *Candida albicans* and *Candida glabrata* encode GDP-mannose pyrophosphorylase. *Biochem Biophys Acta* 2000; 1475: 265-72.

Sambrook J, Russell DW. Molecular cloning (A laboratory manual). 3rd ed. New York: Coldspring Harbor Laboratory Press, 2001.

Saubolle MA. The clinical microbiology laboratory in the diagnosis and monitoring of opportunistic infections in HIV-infected persons. Conference on the Laboratory Science of HIV, 1998: 141-51.

Stateva LI, Oliver SG, Trueman LJ, Venkov PV. Cloning and characterization of a gene which determines osmotic stability in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1991; 11: 4235-43.

Sukroongreung S, Eampokalap BM, Tansuphaswadikul S, Nilakul C, Tantimavanich S. Recovery of *Cryptococcus neoformans* from the nasopharynx of AIDS patients. *Mycopathologia* 1999; 43: 131-4.

Tomlin GC, Hamilton GE, Gardner D, Walmsley RM, Stateva LI,Oliver SG. Suppression of sorbitol dependence in a strain bearing a mutation in the *SRB1/PSA1/VIG9* gene encoding GDP-mannose pyropohosphorylase by *PDE2* overexpression suggests a role for the Ras/cAMP signal-transduction pathway in the control of yeast cell-wall biogenesis. *Microbiology* 2000; 146: 2133-46.

Warit S. Cloning and characterisation of the *Candida albicans* homologue of *SRB1*/*PSA1*/*VIG9*, the essential gene encoding GDP-mannose pyrophosphorylase in *Saccharomyces cerevisiae*. Manchester: University of Manchester Institute of Science and Technology (UMIST), UK, 1999: 251 pp. PhD thesis.

Warit S, Walmsley RM, Stateva LI. Cloning and sequencing of the *Candida albicans* homologue of *SRB1*/*PSA1*/*VIG9*, the essential gene encoding GDP-mannose pyrophosphorylase in *Saccharomyces cerevisiae*. *Microbiology* 1998; 144: 2417-26.

Warit S, Zhang N, Short A, Walmsley RM, Oliver SG, Stateva LI. Glycosylation deficiency phenotypes resulting from depletion of GDP-mannose pyrophosphorylase in two yeast species. *Mol Microbiol* 2000; 36: 1156-66.

Will EA, Roberts IS, Del Poeta M, *et al*. Identification and characterization of the *Cryptococcus neoformans* phosphomannose isomerase-encoding gene, *MAN1*, and its impact on pathogenicity. *Mol Microbiol* 2001; 40: 610-20.

Yoda K, Kawada T, Kaibara C, *et al*. Defect in cell wall integrity of the yeast *Saccharomyces cerevisiae* caused by a mutation of the GDP-mannose pyrophosphorylase gene *VIG9*. *Biosci Biotechnol Biochem* 2000; 64: 1937-41.

Zakrewska A, Palamarczyk G, Krotkiewski H, *et al*. Overexpression of the gene encoding GTP:mannose-1-phosphate guanyltransferase, *mpg1*, increases cellular GDP-mannose levels and protein mannosylation in *Trichoderma reesei*. *Appl Environ Microbiol* 2003; 69: 4383-89.

Zhang N, Gardner DCJ, Oliver SG, Stateva LI. Down-regulation of the expression of PKC1 and *SRB1*/*PSA1*/*VIG9*, two genes involved in cell wall integrity in *Saccharomyces cerevisiae*, causes flocculation. *Microbiology* 1999a; 145: 309-16.

Zhang N, Gardner DCJ, Oliver SG, Stateva LI. Genetically controlled cell lysis in the yeast *Saccharomyces cerevisiae*. *Biotechnol Bioeng* 1999b; 64: 607-15.